Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

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Iterative in situ Click Chemistry Creates Antibody-Like Protein Capture Agents

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MATERIALS

Fmoc-D-**X**-OH (Fmoc, fluoren-9-ylmethoxycarbonyl) (**X** = Ala, Arg(Pbf) (Pbf, pentamethyldihydrobenzofuran-5-sulfonyl), Asn(Trt) (Trt, trityl), Asp(O*t*Bu) (*t*Bu, *tert*-butyl), Glu(OtBu), Gln(Trt), Gly, His(Trt), Ile, Leu, Lys(Boc) (Boc, *tert*-butyloxycarbonyl), Met, Phe, Pro, Ser(*t*Bu), Thr(*t*Bu), Trp(Boc), Tyr(*t*Bu), and Val) were purchased (Anaspec; San Jose, CA) and used as received. TentaGel S-NH_2 resin (90 μm, 0.31 mmol/g) (Rapp-Polymere; Tübingen, Germany) were utilized for OBOC library construction. Amino acid coupling reactions were performed in 1-methyl-2-pyrrolidinone (NMP, 99%) with HATU (2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate, ChemPep; Miami, FL) and *N,N*′-diisopropylethylamine (DIEA). For removal of N^{α}-Fmoc protecting groups, a solution of 20% piperidine in NMP was used. For final deprotection of the peptide libraries, trifluoroacetic acid (TFA, 98% min. titration) and triethylsilane (TES) were used. All solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used as received, unless otherwise noted.

OBOC libraries were synthesized using a 180-degree variable-speed shaker, fitted with small sample adapter (St. John Associates; Beltsville, MD). Fritted polypropylene solid-phase synthesis tubes were used for repeated split-mix cycles. A 24-port SPE vacuum manifold system (Grace, Deerfield, IL) was used for exchanging coupling solutions and washing the resins.

Fmoc-D-propargylglycine (**Fmoc-D-Pra-OH**) was acquired (Chem-Impex International; Wood Dale, IL) and used as the acetylene handle for construction of ligands.

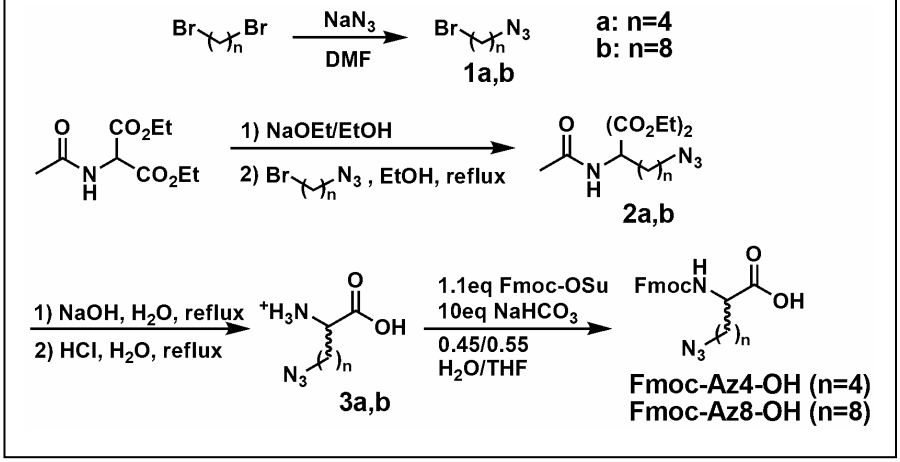
Proteins. Bovine carbonic anhydrase II (bCAII, C2522), from bovine erythrocytes, lyophilized powder, was obtained (Sigma-Aldrich; St. Louis, MO) and used as received. To prepare the protein for screening, dye-labeling was accomplished with the Alexa Fluor 647 Microscale Protein Labeling Kit (Invitrogen; Carlsbad, CA) following the manufacturer's protocol for a low degree of labeling (DOL). Protein (100 μg) was incubated with 6 mol equiv Alexa Fluor 647 succinimidyl ester for 15 min at 25 °C. Excess dye was removed by BioGel P-6 size exclusion resin (Bio-Rad, Hercules, CA). The labeled protein (bCAII-Alexa Fluor 647) was characterized by UV-Vis and mass spectrometry.

Human carbonic anhydrase II (hCAII, C6165), from human erythrocytes, lyophilized powder, was obtained (Sigma-Aldrich; St. Louis, MO) and used in affinity and selectivity studies. Both bCAII and hCAII were tested by SDS gel electrophoresis, and confirmed to display a single band corresponding to 29,000 Da.

ARTIFICIAL AMINO ACID SYNTHESIS (SCHEME S1)

Azidobutylbromide (1a). To a solution of 1,4-dibromobutane (123 mmol), sodium azide (61.5 mmol) was added and stirred overnight in *N,N′*-dimethylformamide (DMF) at 50 °C. The reaction was diluted with ethyl acetate, and the organic layer was washed with water, then brine, and then dried over $MgSO_4$. The crude residue was purified by silica gel chromatography (100% hexanes) to give a product (80%) as a clear oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.44 (2H, t, J = 6.3 Hz), 3.34 (2H, t, J = 6.6 Hz), 1.93-1.98 (2H, m), 1.74-1.79 (2H, m).

SCHEME S1



Azidooctylbromide (1b). Synthesis was carried out as described above, except 1,8-dibromooctane was used as the starting material. ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.41 (2H, t, J = 6.9 Hz), 3.26 (2H, t, J = 6.6 Hz), 1.86 (2H, p, J = 6.9 Hz), 1.60 (2H, p, J = 8.7 Hz), 1.34-1.55 (4H, m).

Diethyl 2-acetamido-2-(4-azidobutyl)malonate (2a). To a solution of 0.598 g (0.026 mol) sodium metal in 25 mL absolute EtOH, 5.65 g diethyl acetamidomalonate (0.026 mol) was added, following previously published procedures.[1] The mixture was stirred for 30 min at room temperature. By dropwise addition, azidobutylbromide **1a** (4.82 g, 0.027 mol) was added with stirring. The reaction mixture was stirred for 2 h at room temperature and refluxed for 6 h at 80 °C. After cooling overnight, the reaction mixture was concentrated to dryness, and the residue was extracted with diethyl ether. The combined ether extracts were washed with water, sat. $NaHCO_3$, water, and brine, and were dried over $MgSO_4$ and then concentrated. Silica gel chromatography (Hex:EtOAc = 1:1) gave a product (63%) as a clear, viscous oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.77 (1H, s), 4.24 (4H, q, *J* = 6.9 Hz), 3.26 (2H, t, *J* = 6.9 Hz), 2.31-2.37 (2H, m), 2.04 (3H, s), 1.59 (2H, p, *J* = 7.5 Hz), 1.26 (6H, t, *J* = 6 Hz), 1.16-1.27 (2H, m). ESI-MS *m/e* 315.

Diethyl 2-acetamido-2-(4-azidooctyl)malonate (2b). Similar synthetic protocol as **2a** was adopted, only azidooctylbromide **1b** served as the starting material. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.76 (1H, s), 4.24 (4H, q, *J* = 7.2 Hz), 3.24 (2H, t, *J* = 6.9 Hz), 2.27-2.33 (2H, m), 2.04 (3H, s), 1.56 (2H, p, *J* = 7.5 Hz), 1.25 (6H, t, *J* = 7.2 Hz), 1.06-1.16, 1.2-1.4 (10H, m). ESI-MS *m/e* 371.

2-Azidobutyl amino acid (3a). Following standard methods,[2] the diester **2a** (2.8 mmol) in 25 mL of 10% NaOH solution was heated to reflux for 4 h. The solution was then neutralized with concentrated HCl and evaporated. The residue was dissolved in 25 mL of 1 M HCl and heated to reflux for 3 h. The solvent was reduced and extraction with MeOH afforded amino acid **3a** as the hydrochloride salt (85%). ^{1}H NMR (300 MHz, CD_3OD): δ 3.98 (1H, t, *J* = 6.3 Hz), 3.35 (2H, t, *J* = 7.8 Hz), 1.45-1.7, 1.85-2.05 (6H, m). MALDI-MS *m/e* 173.

2-Azidooctyl amino acid (3b). Synthesis was carried out as described above, using diester **2b** as the starting material. ^{1}H NMR (300 MHz, CD_3OD): δ 3.94 (1H, t, *J* = 6.3 Hz), 3.27 (2H, t, *J* = 6.9 Hz), 1.3-1.52, 1.52-1.62, 1.8-1.98 (14H, m). ESI-MS *m/e* 229.

Fmoc-2-Azidobutyl amino acid (Fmoc-Az4-OH). The amino acid **3a** (26.3 mmol) was dissolved in 0.45:0.55 H_2O:THF (150 mL), and $NaHCO_3$ (22.1 g, 263 mmol) was added, following published methods.[3] After the mixture was cooled to 0 °C, Fmoc-OSu (9.7 g, 28.9 mmol) was added dropwise over 5 min. The reaction mixture was allowed to come to room temperature and stirred overnight. Evaporation of THF was completed *in vacuo* and the aqueous

residue was washed with diethyl ether (2 × 200 mL). The aqueous layer was then collected and acidified with conc. HCl to pH 2 before extraction with ethyl acetate (4 × 100 mL). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered, and concentrated. The organic residue was purified by column chromatography (2% MeOH in DCM) to yield a white powder (48% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.76 (2H, d, *J* = 7.5 Hz), 7.59 (2H, d, *J* = 6.9 Hz), 7.40 (2H, t, *J* = 7.5 Hz), 7.31 (2H, t, *J* = 7.5 Hz), 5.34 (1H, d, *J* = 7.8 Hz), 4.49-4.59 (1H, m), 4.43 (2H, d, *J* = 6.6 Hz), 4.22 (1H, t, *J* = 6.6 Hz), 3.27 (2H, t, *J* = 6.6 Hz), 1.3-2.0 (6H, m). ESI-MS *m/e* 395.

Fmoc-2-Azidooctyl amino acid (Fmoc-Az8-OH). The amino acid **3b** was treated to Fmoc protection as described above. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.75 (2H, d, *J* = 7.5 Hz), 7.57-7.61 (2H, m), 7.39 (2H, t, *J* = 7.5 Hz), 7.30 (2H, t, *J* = 7.2 Hz), 5.40 (1H, d, *J* = 8.1 Hz), 4.42-4.52 (1H, m), 4.40 (2H, d, *J* = 7.2 Hz), 4.21 (1H, t, *J* = 7.2 Hz), 3.23 (2H, t, *J* = 6.9 Hz), 1.18-1.98 (14H, m). ESI-MS *m/e* 450.

PEPTIDE LIBRARY CONSTRUCTION

Randomized OBOC libraries of penta- to heptapeptides were synthesized manually via standard split-and-mix solid-phase peptide synthesis methods on 90 μm polyethylene glycol-grafted polystyrene beads (TentaGel S-NH_2, 0.31 mmol/g, 2.86 x 10^6 beads/g).[4-6] Non-natural D-stereoisomers (denoted by lowercase one-letter amino acid code) were used at every possible position in the peptide sequence. At least a 5-fold excess of beads was utilized in each library synthesis to ensure adequate representation of each library element. A standard solid-phase peptide synthesis method with Fmoc chemistry was used.[7] All wash, deprotection, and coupling steps were facilitated by 180-degree shaking of the resin. The resin was pre-swelled in NMP in a plastic fritted reaction vessel, and was separated into multiple aliquots. Each aliquot was reacted with 2-fold molar excess (relative to resin) of a single N^{α}-Fmoc-amino acid. Amide coupling was initiated by addition of a 2-fold molar excess of HATU and a 6-fold molar excess of DIEA.[8] The coupling reaction was run for 15 min. Another 2 equiv N^{α}-Fmoc-amino acid, 2 equiv HATU, and 6 equiv DIEA were added, and allowed to react for 15 min ("double coupling"). In some cases, "triple coupling" with a third set of coupling reagents and N^{α}-Fmoc-amino acid was performed (Table S1, **Libraries D**, **E**, **F**, and **G**). Following coupling, the aliquots were thoroughly washed (5 × NMP), mixed together into a single vessel, and

Table S1. Libraries used in this study.[†]

	Formula	Components	# of unique sequences
A	$x_1x_2x_3x_4x_5$	x_i = 19 D-amino acids (no D-Cys)	2,476,099
B	$x_1x_2x_3x_4x_5x_6$	x_i = r, k, l, w, f, h, y	117,649
C	**Az_n**-$x_2x_3x_4x_5x_6$-**Az_n**	x_i = 19 D-amino acids (no D-Cys) **Az_n** = 1/3 **Az4**, 1/3 **Az8**, 1/3 nothing	22,284,891
D	$x_1x_2x_3x_4x_5x_6$-**Tz1**-kfwlkl	x_i = k, l, w, f, i, g, v	117,649
Tz1 = triazole formed between **Az4** (on terminal k) and **D-Pra** (on x_6)			
E	$x_7x_6x_5x_4x_3x_2$-**Tz2**-kwlwGl-**Tz1**-kfwlkl	x_i = d, r, s, w, G, f, l	117,649
Tz1 = triazole formed between **Az4** (on terminal k) and **D-Pra** (on l) **Tz2** = triazole formed between **Az4** (on terminal x_2) and **D-Pra** (on k)			
F	**Az4**-$x_2x_3x_4x_5x_6x_7$	x_2 = r, n, l, i; x_3 = w, f, l, i; x_4 = r, w, f, l, i; x_5 = w, f, v, l; x_6 = r, w, f, l, k; x_7 = f, r	3200
G	$x_7x_6x_5x_4x_3x_2$-**Tz2**-kwlwGl-**Tz1**-kfwlkl		3200

[†] Randomized positions are denoted by x (for D-amino acids) and **Az_n** (for azide-containing artificial amino acids).

deprotected with 20% piperidine in NMP (30 min). The resin was thoroughly washed (5 × NMP), dried (5 × DCM), and re-divided into multiple equal-mass aliquots for the next cycle of coupling. The procedures were repeated until the desired length of peptide was attained.

The amino acid side-chain protecting groups were then removed by incubation in trifluoroacetic acid (95%), water (5%), and triethylsilane (2-fold molar excess per protected side chain) for 2 h at 25 °C. The library resin was then neutralized with DMF, and washed thoroughly with DMF (5 ×), water (5 ×), methanol (MeOH, 5 ×), and methylene chloride (DCM, 5 ×),[9] and then dried under vacuum and stored in phosphate-buffered saline [PBS (pH 7.4)] + 0.05% NaN_3 at 25 °C.

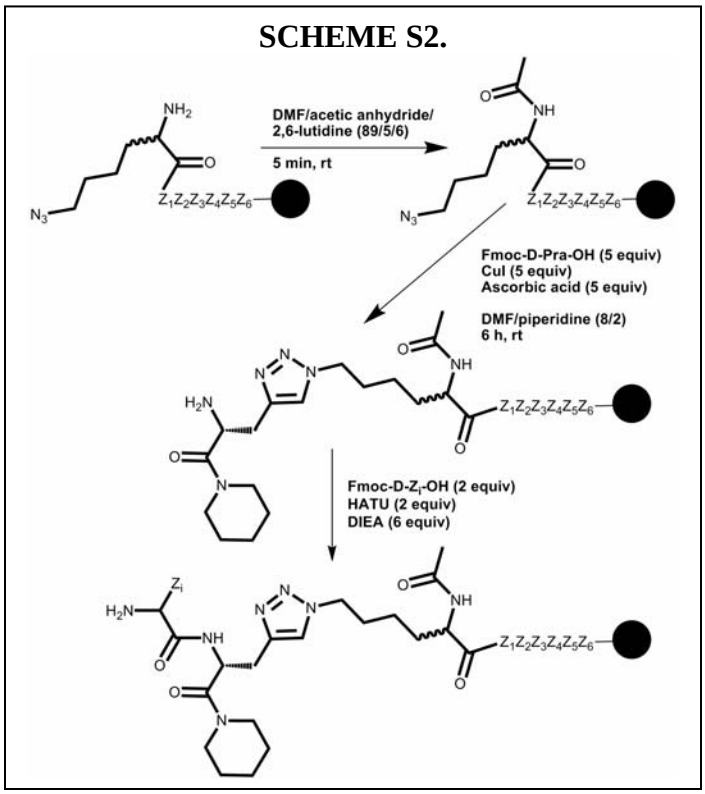
BULK PEPTIDE SYNTHESIS

Bulk synthesis of hit peptide sequences was performed on either Fmoc-Rink amide MBHA (50 μm, 0.67 mmol/g) or 2-chlorotrityl chloride (1.5 mmol/g) resins (Anaspec; San Jose, CA), on a typical resin scale of 0.3 g per sequence. Crude peptides were precipitated with ether, and then purified to >98% by HPLC (Beckman Coulter System Gold 126 Solvent Module and 168 Detector, Fullerton, CA) on a C_{18} reversed phase semi-preparative column (Phenomenex Luna 10 μm, 250 × 10 mm). The pure peptides were used for affinity measurements, screens, and binding assays. Hit peptide sequences were also re-synthesized on TentaGel S-NH_2 on a similar resin scale, and used for on-bead binding assays.

ON-BEAD CLICK REACTION

For preparing **Libraries D, E,** and **G** (Table S1), as well as for bulk synthesis of biligand and triligand candidates, the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was carried out on bead, with 4 general steps: (1) anchor ligand synthesis, (2) acetylation, (3) click reaction, and (4) addition of 2° ligand sequence. Scheme S2 illustrates the acetylation and click reactions for a 6-mer peptide (Z = any amino acid). The fully protected TentaGel S-NH_2 bead-bound anchor ligand (0.420 g, 0.13 mmol) was capped by a solution of acetic anhydride (1 mmol) in 2,6-lutidine and DMF.[10] The acetylated peptide was reacted with **Fmoc-D-Pra-OH** (0.218 g, 0.65 mmol) in the presence of CuI (0.124 g, 0.65 mmol), L-ascorbic acid (0.114 g, 0.65 mmol), and DMF/piperidine (8/2) at 25 °C for 6 h.[11] The resin was washed with 5 × 5 mL $Et_2NCSSNa•3H_2O$ (sodium diethyldithiocarbamate trihydrate, 1% w/v), containing 1% DIEA (v/v) in DMF to remove the coordinated copper from click reaction.[12]

SCHEME S2.



The biligand anchor **(D-Pra)-kwlwGl-Tz1-kfwlkl** was synthesized on 2-

chlorotrityl chloride (1.6 mmol/g) resin (Anaspec, San Jose, CA) using Scheme S2. The biligand anchor was released either as the fully deprotected peptide by cleavage with 95:5 TFA:water (+ 2 mol equiv TES per side chain protecting group), or as the fully protected peptide by cleavage with 99:1 DCM:TFA.[13] To facilitate the on-bead click reaction, it is noted that the 1° ligand was synthesized here as **Az4-kfwlkl** (displaying N-terminal Az_n modification), and to this sequence was coupled D-Pra and the 2° ligand to produce the linear biligand.

Triligands were synthesized by click reaction between the fully protected biligand anchor (0.274 g, 0.1 mmol, >98% HPLC) and bead-bound 3° ligand **Az4-nlivfr** (0.1 g, 0.03 mmol) using CuI (0.021 g, 0.1 mmol) and L-ascorbic acid (0.020 g, 0.1 mmol) in DMF/piperidine (8/2).

TYPICAL SCREENING PROCEDURES

A typical screen began with a library incubation in PBS (pH 7.4) + 0.1% Tween 20 + 0.1% bovine serum albumin (BSA) + 0.05% NaN_3 (PBSTBNaN_3) for 1 h, with shaking, to block non-specific protein binding.[14] The library was then washed with 3 × 5 mL PBSTBNaN_3. On-bead multi-ligand screens were conducted at an appropriate bCAII-Alexa Fluor 647 dilution (Table S2), and then washed with 3 × 5 mL PBSTBNaN_3, 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and finally 6 × 5 mL PBS (pH 7.4). All in situ screens contained an additional 2 h pre-incubation of bCAII-Alexa Fluor 647 with anchor ligand (≥2000 equiv, relative to protein), after which the bead library was added to this mixture and the screen was continued (Table S2). Following in situ screening, beads were washed with 3 × 5 mL PBSTBNaN_3, 3 × 5 mL PBS (pH 7.4) + 0.1% Tween 20, and then 6 × 5 mL PBS (pH 7.4).

Screened beads were transferred onto a glass microscope slide and immediately imaged for fluorescence using a GenePix 4200 array scanner (λ_{ex} = 635 nm). The hit beads were selected manually by glass micropipette. To remove bound proteins, each hit bead was incubated in 7.5 M guanidine hydrochloride (pH 2.0) for 1 h, followed by ten rinses with water.

Edman sequencing of single hit beads was carried out on a Model 494 Procise cLC Sequencing System (Applied BioSystems, Foster City, CA). Iterative N-terminal chemical degradation cycles yielded direct positional amino acid information. Each degradation cycle produced one PTH-amino acid (PTH = phenylthiohydantoin) product that was analyzed by HPLC and identified by retention time as compared with PTH-amino acid standards.

Table S2. Screening summary. All screens at pH = 7.4 and T = 25 ^{o}C, unless otherwise noted.

Screen	Library	[bCAII-AF647]	Time(h)	% hit beads	Buffer	Other components
An1	**A**	100 nM	1 h	0.02%	PBS	
An2a	**B**	50 nM	1 h	0.09%	PBS	
An2b	**B**	8 nM	24 h	2 hits	PBS	
Bi1	**C**	50 nM	2 h; 37^{o} (no beads) + 48 h; 37^{o}	0.007%	PBS + 1% DMSO (v/v)	100 μM of **lklwfk-(D-Pra)**
Bi2a	**D**	50 nM	17 h	0.07%	PBSTBNaN_3	
Bi2b	**D**	10 nM	17 h	0.008%	PBSTBNaN_3	
Tri1	**C**	10 nM	2 h (no beads) +15 h	0.007%	PBSTBNaN_3 + 1% DMSO (v/v)	100 μM of **(D-Pra)-kwlwGl-Tz1-kfwlkl**
Tri2	**E**	10 nM	17 h	0.008%	PBSTBNaN_3	
TriX	**A**	10 nM	17 h	0.007%	PBSTBNaN_3 + 1% DMSO (v/v)	100 μM of **(D-Pra)-kwlwGl-Tz1-kfwlkl**
Tri3	**F**	0.5 nM	2 h (no beads) +18 h	0.005-0.01%	PBSTBNaN_3 + 1% DMSO (v/v)	100 μM of **(D-Pra)-kwlwGl-Tz1-kfwlkl**
Tri4	**G**	0.25 nM	18 h	0.005-0.01%	PBSTBNaN_3	

COMPLETE HIT SEQUENCING RESULTS: Tables S3-S13

Table S3. First-generation anchor ligand screen **An1** results.

	x_1	x_2	x_3	x_4	x_5
hit1	r	r	y	h	r
hit2	m/v	r	w	k	r
hit3	k	r	w	y	y
hit4	w	k	k	k	w
hit5	h	f	f	f	r
hit6	s	r	--	r	r
hit7	r	r	w	h	y
hit8	r	k	w	w	w
hit9	r	w	s	f	r
hit10	r	r	g	w	r
hit11	g	f	r	r	w
hit12	r	t	r	r	w
hit13	m	r	w	k	r
hit14	y	r	k	r	w
hit15	a	--	--	--	--
hit16	r	r	i	r	w
hit17	--	--	k/l	w	--
hit18	r	w	--	--	r
hit19	k/l	r	--	w	r
hit20	w	r	f	r	y
hit21	d/p	y	y	r	r
hit22	r	y	w	k	k
hit23	k/l	r	r	r	w
hit24	y	r	r	k	w
hit25	r	k/l	f	y	r
hit26	r	w	w	k	r

	x_1	x_2	x_3	x_4	x_5
hit27	w	r	--	y	r
hit28	h	r	w	r	r
hit29	w	y	r	k	r
hit30	l	r	f	r	r
hit31	w	k	r	k	k
hit32	r	r	r	w	s/m
hit33	r	r	k	f	w
hit34	r	r	w	r	y
hit35	w	r	h	y	k
hit36	r	r	y	f	r
hit37	w	r	k	w	r
hit38	w	y	--	r	r
hit39	y	r	r	r	h
hit40	y	r	r	r	w
hit41	p	f	y	w	r
hit42	k	y	w	r	k
hit43	r	y	w	h	k
hit44	r	w	h	w	n
hit45	r	h	f	h	h/f
hit46	r	r	--	h	r
hit47	r	y	r	r	r
hit48	y	f	h	h/w	w
hit49	r	r	r	w	y
hit50	w	r	r	r	r/--
hit51	r	w	k	f	h

Table S4. Second-generation anchor ligand screen **An2a** results.

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	y	r	w	f	k	f
hit2	h/r	h/r	f	l	l/r	r
hit3	f	r	f	y	y	r
hit4	h/r	f	f	k	l	--
hit5	k	l	f	l	k	l
hit6	l	f	l	w	l	k
hit7	f	f	f	r	y	--
hit8	h/r	f	f	f	r	--
hit9	r	w	w	l	k	f
hit10	h/r	f	f	r	y	y
hit11	l	k	l	f	l	k
hit12	f	r	r	w	w	k
hit13	h/r	y	f	f	k	l
hit14	l	k	f	f	f	k
hit15	h/r	f	f	r	r	--

Table S5. Second-generation anchor ligand screen **An2b** results.

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	h	l	y	f	l	r
hit2	l	k	l	w	f	k

Table S6. In situ biligand screen **Bi1** results.

	Az_n	x_2	x_3	x_4	x_5	x_6	Az_n
hit1	**Az4**	k	i	w	i	G	
hit2	**Az8**	r	l	w	v	G	**Az4**
hit3	**Az8**	r	r	r	k	r	**Az8**
hit4	**Az4**	l	l	v	i	k	**Az4**
hit5	**Az4**	m	i	l	i	k	
hit6	**Az8**	i	i	i	m	r	**Az4**
hit7	**Az8**	i	i	i	w	r	**Az8**
hit8	**Az4**	n	v	i	i	f	
hit9	**Az4**	i	f	l	v	k	**Az8**
hit10	**Az4**	k	i	w	i	G	**Az8**
hit11	**Az4**	r	r	k	f	r	**Az8**
hit12	**Az4**	r	v	w	l	r	**Az8**
hit13	**Az8**	k	y	r	r	r	**Az4**
hit14	**Az8**	r	r	k	v	w	**Az4**
hit15	**Az4**	i	f	l	v	k	**Az8**
hit16		k	r	k	r	f	**Az4**
hit17	**Az8**	k	i	w	i	k	
hit18	**Az8**	y	r	k	f	k	
hit19	**Az4**	i	f	f	r	v	**Az8**
hit20		a	r	k	k	y	**Az4**
hit 21		r	k	r	t	i	**Az4**
hit 22	**Az8**	k	m	v	f	k	**Az4**
hit23	**Az4**	l	i	m	k	i	**Az4**

Table S7. On-bead biligand screen **Bi2a** results.

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	f	k	l	w	i	k
hit2	v	w	l	w	G	G
hit3	f	w	f	w	G	G
hit4	k	w	f	w	G	G
hit5	f	k	l	w	l	k
hit6	k	w	f	w	G	G
hit7	w	w	i	w	G	G
hit8	k	G	w	l	w	G
hit9	k	l	w	i	w	G
hit10	l	w	i	w	G	l
hit11	f	k	G	f	l	i
hit12	f	w	i	w	G	k
hit13	l	w	l	w	G	i
hit14	i	i	v	l	w	k
hit15	l	i	i	f	v	
hit16	v	k	f	i	l	l
hit17	l	G	f	f	w	i
hit18	k	k	l	k	k	l
hit19	f	k	l	w	i	k
hit20	w	i	w	G	G	f
hit 21	f	f	l	l	v	k
hit 22	k	f	k	f	w	k
hit23	l	i	k	l	f	v
hit24	l	w	f	w	G	v
hit25	f	w	f	w	G	i
hit26	G	w	f	w	G	v
hit27	G	w	i	w	G	k

Table S8. On-bead biligand screen **Bi2b** results.

	x_1	x_2	x_3	x_4	x_5	x_6
hit1	k	w	i	w	G	w
hit2	k	w	i	w	G	v
hit3	k	w	l	w	G	l
hit4	k	w	i	w	G	l
hit5	k	w	i	w	G	w
hit6	k	w	l	w	G	l
hit7	G	w	i	w	G	i
hit8	k	i	f	k	i	f

Table S9. First-generation in situ triligand screen **Tri1** results.

	Az_n	x_2	x_3	x_4	x_5	x_6	Az_n
hit1	**Az4**	n	i	i	i	v	
hit2	**Az4**	i	i	l	l	k	**Az4**
hit3	**Az4**	n	i	i	v	l	
hit4	**Az4**	n	m	i	f	l	**Az4**
hit5	**Az4**	n	v	l	v	l	
hit6	**Az4**	n	l	i	l	f	**Az4**
hit7	**Az4**	n	l	i	l	f	**Az4**
hit8	**Az8**	r	l	w	i	r	**Az4**
hit9	**Az4**	n	l	i	v	f	**Az4**
hit10	**Az4**	r	m	w	v	k	**Az8**
hit11	**Az4**	i	i	l	l	k	**Az8**
hit12	**Az4**	i	l	v	v	r	**Az4**
hit13	**Az4**	n	l	l	f	l	**Az4**
hit14	**Az4**	n	i	i	v	y	
hit15		m	k	r	k	k	**Az8**
hit16	**Az4**	i	l	i	r	w	**Az4**
hit17	**Az8**	i	i	v	f	r	**Az8**
hit18	**Az8**	y	f	t	r	r	
hit19	**Az4**	n	m	i	i	v	**Az4**
hit20	**Az8**	i	l	i	a	k	**Az4**
hit21	**Az4**	i	l	l	r	w	
hit22	**Az8**	i	v	v	f	r	**Az4**
hit23	**Az4**	l	l	l	v	k	**Az4**
hit24	**Az4**	k	v	w	i	k	**Az4**
hit25	**Az4**	i	m	v	l	r	**Az4**

Table S10. First-generation on-bead triligand screen **Tri2** results.

	x_2	x_3	x_4	x_5	x_6	x_7
hit1	r	l	w	l	r	f
hit2	r	l	w	l	r	l
hit3	r	f	f	f	r	f
hit4	r	l	f	l	r	f
hit5	l	f	f	w	f	r
hit6	l	w	f	f	f	r
hit7	l	f	l	w	f	r
hit8	l	w	l	f	f	r
hit9	l	f	f	w	l	r
hit10	r	r	r	l	w	r
hit11	r	l	w	l	r	f
hit12	w	r	r	r	r	w
hit13	r	f	r	f	r	w
hit14	f	w	f	f	w	r

Table S11. Second-generation in situ triligand screen **Tri3** results.

	x_2	x_3	x_4	x_5	x_6	x_7
hit1	n	l	i	v	f	r
hit2	n	l	i	v	l	r
hit3	n	i	i	l	l	r
hit4	i	l	f	l	f	r
hit5	n	l	i	v	l	r
hit6	n	i	i	l	w	r
hit7	n	l	i	v	f	r
hit8	n	l	i	v	f	r

Table S12. Second-generation on-bead triligand screen **Tri4** results.

	x_2	x_3	x_4	x_5	x_6	x_7
hit1	n	l	i	v	f	r
hit2	n	l	i	v	f	r
hit3	n	i	i	v	f	r
hit4	n	i	i	v	f	r
hit5	n	i	i	l	l	r
hit6	n	l	i	v	l	r
hit7	n	l	i	v	f	r

Table S13. Azide-free in situ triligand screen **TriX** results (control).

	x_1	x_2	x_3	x_4	x_5
hit1	w	f	r	r	r
hit2	s	w	v	w	G
hit3	p	v	y	f	w
hit4	d	d	y	w	G
hit5	i	w	a	y	w
hit6	d	n	w	G	f
hit7	a	w	w	a	t
hit8	r	f	r	r	f
hit9	d	w	w	h	t
hit10	r	f	r	w	r
hit11	d	e	w	p	h
hit12	a	w	w	l	w
hit13	a	w	w	a	y
hit14	d	k	k	i	y
hit15	d	w	s	i	e
hit16	s	w	w	f	y
hit17	d	w	l	r	y
hit18	s	w	a	f	y
hit19	d	l	f	l	w
hit20	d	w	a	t	w
hit21	f	k	y	r	s
hit22	d	q	r	w	r
hit23	i	w	s	t	h
hit24	l	i	v	m	w

BILIGANDS AND TRILIGANDS REPORTED: Figures S1-S6

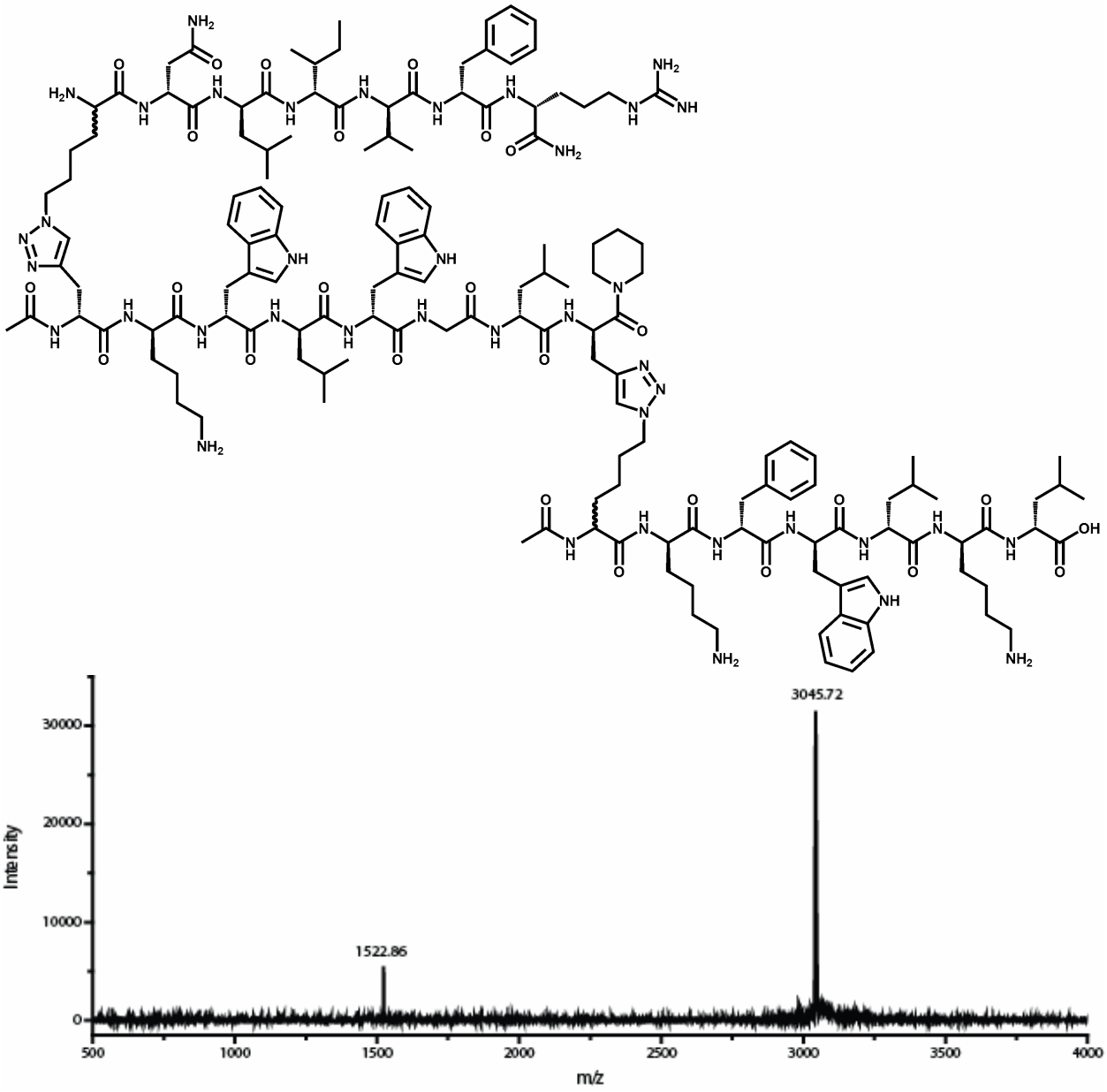


Figure S1. Triligand (Mol. Wt. 3045.72), used in SPR measurement of affinity.

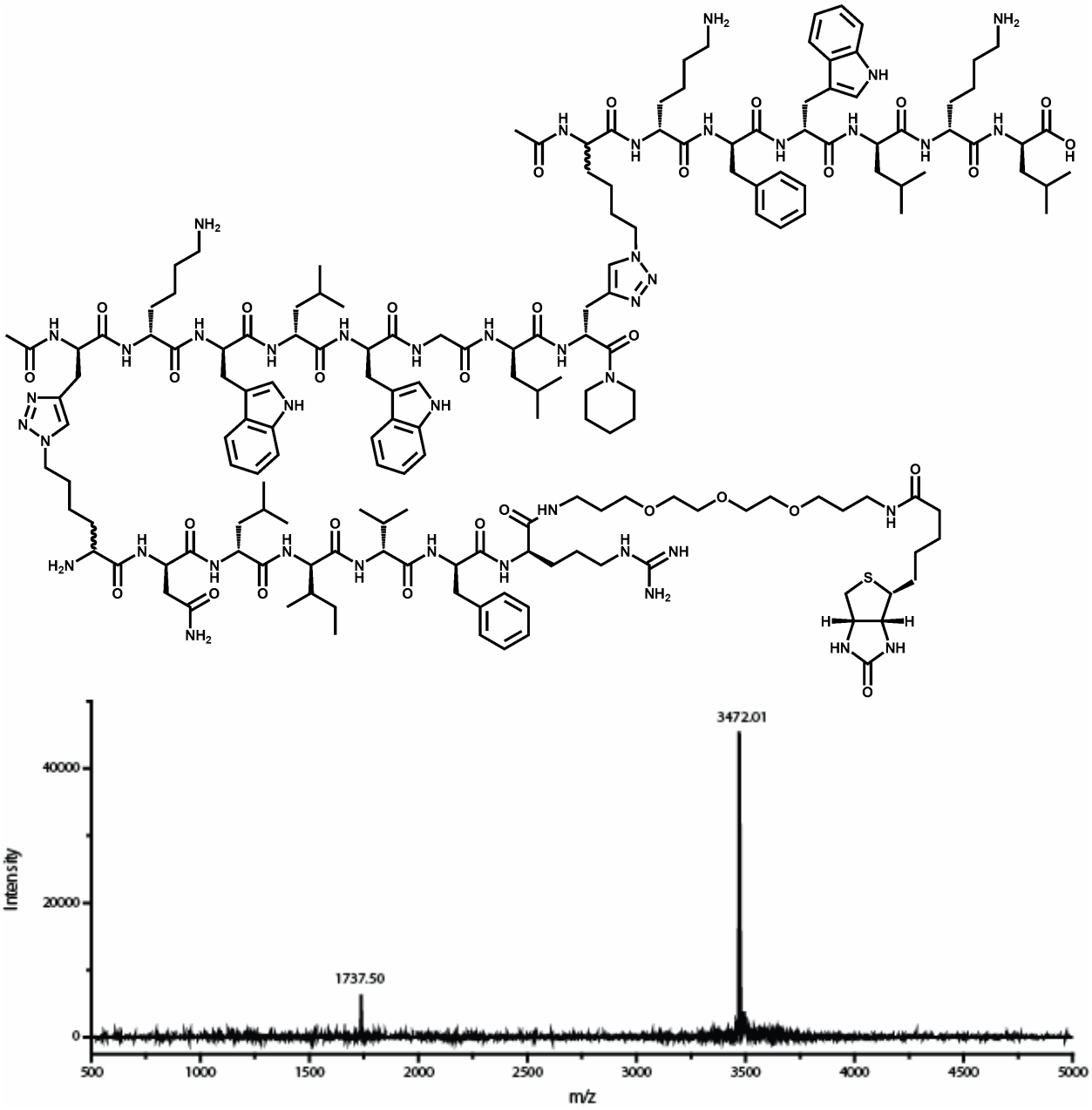


Figure S2. Triligand, biotin conjugate (Mol. Wt. 3475.29), used in dot blot experiments.

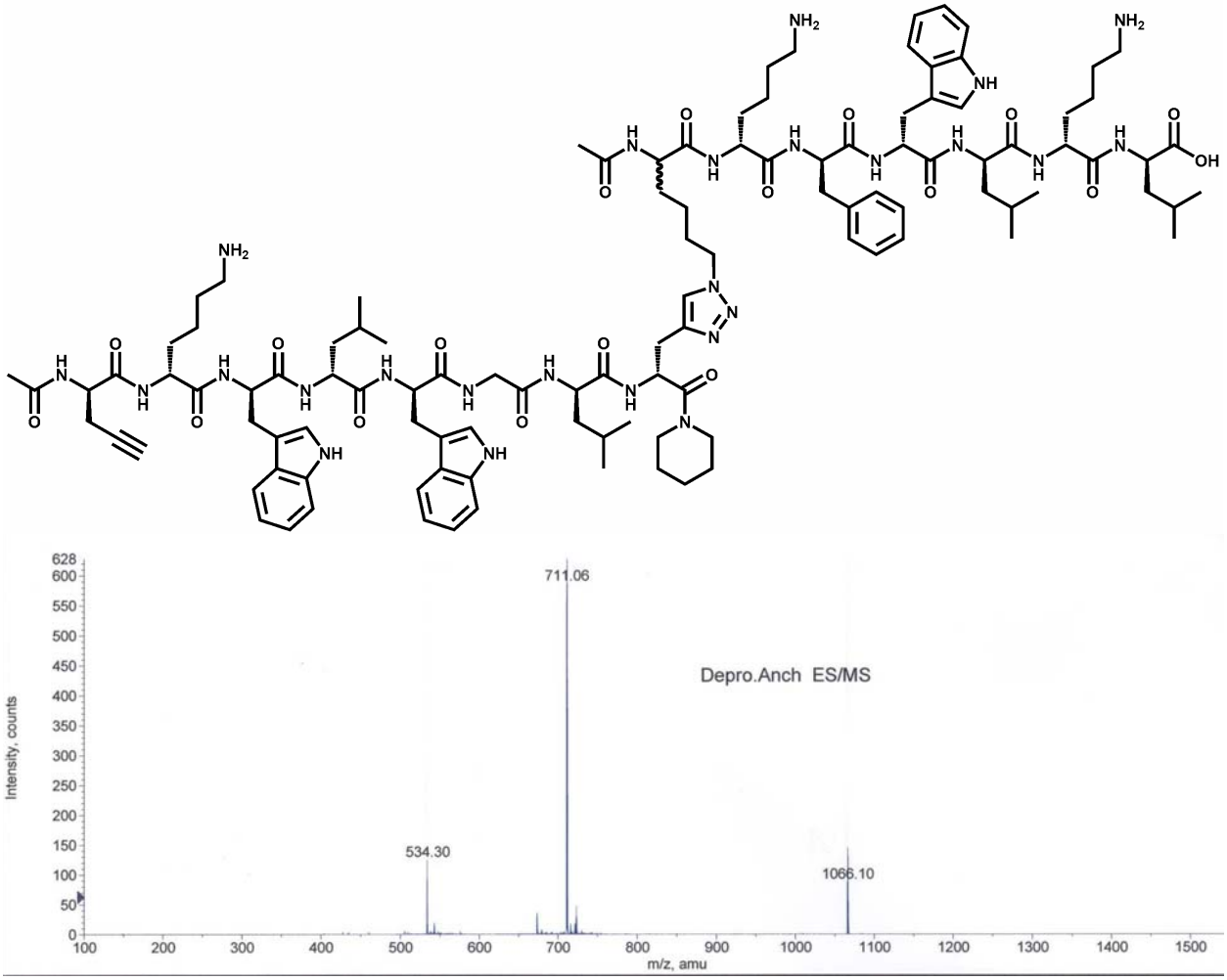


Figure S3. Biligand Anchor, deprotected (Mol. Wt.: 2131.61), used for in situ screens.

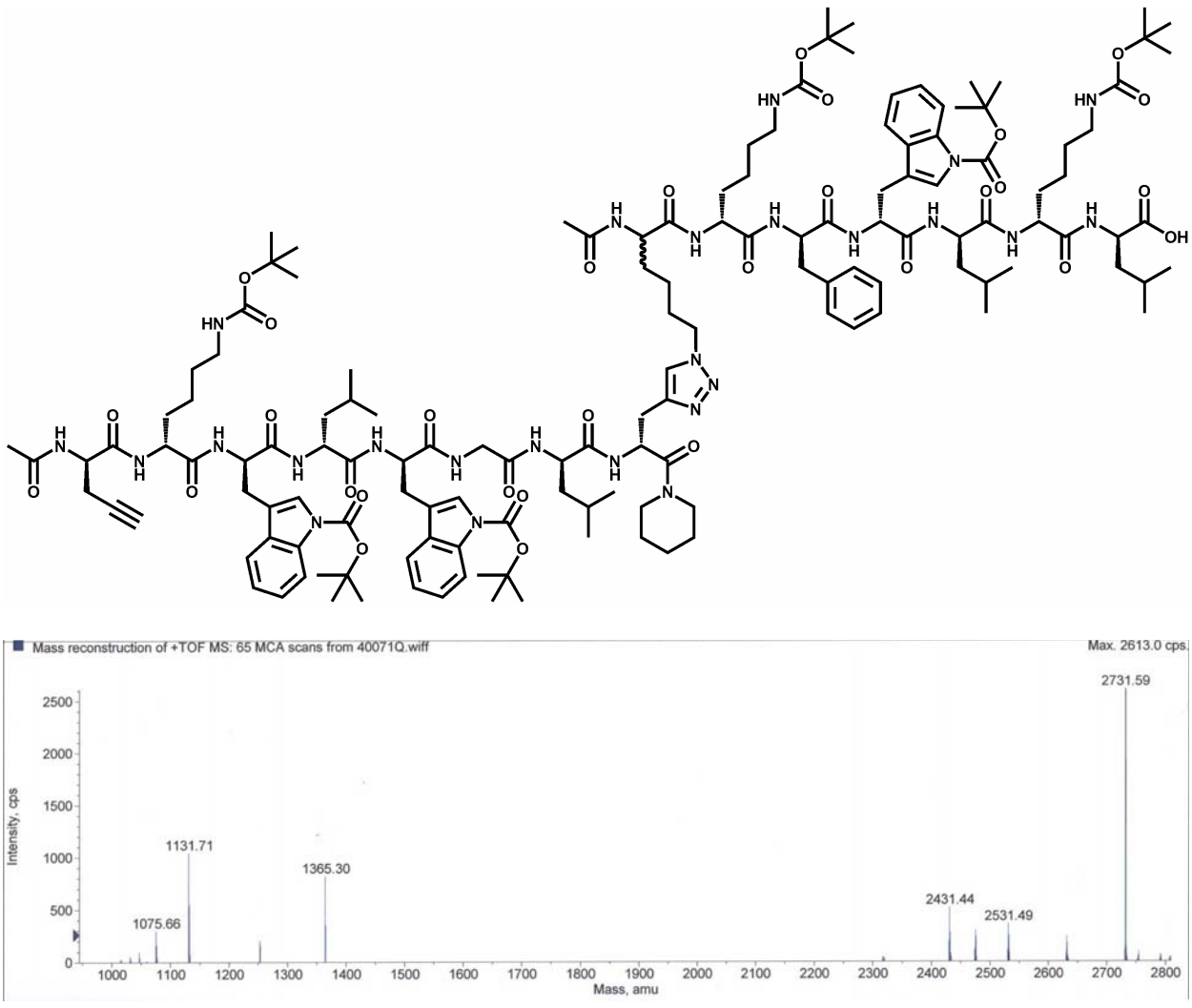


Figure S4. Biligand Anchor, fully protected (Mol. Wt.: 2732.30), used in bulk triligand synthesis.

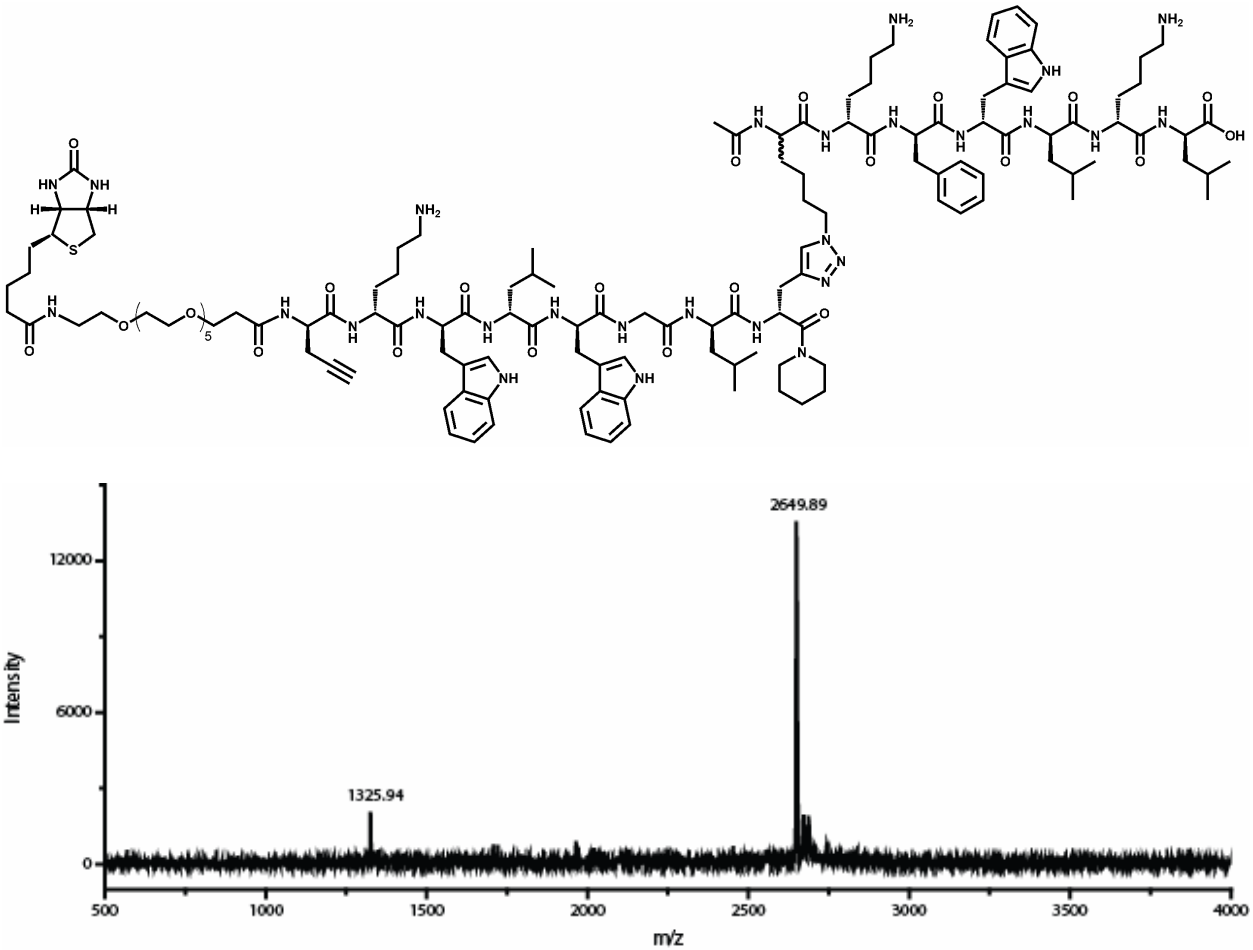


Figure S5. Biligand Anchor, biotin conjugate (Mol. Wt.: 2651.26), used in dot blot experiments and assays for detecting on-bead, protein-templated multi-ligand.

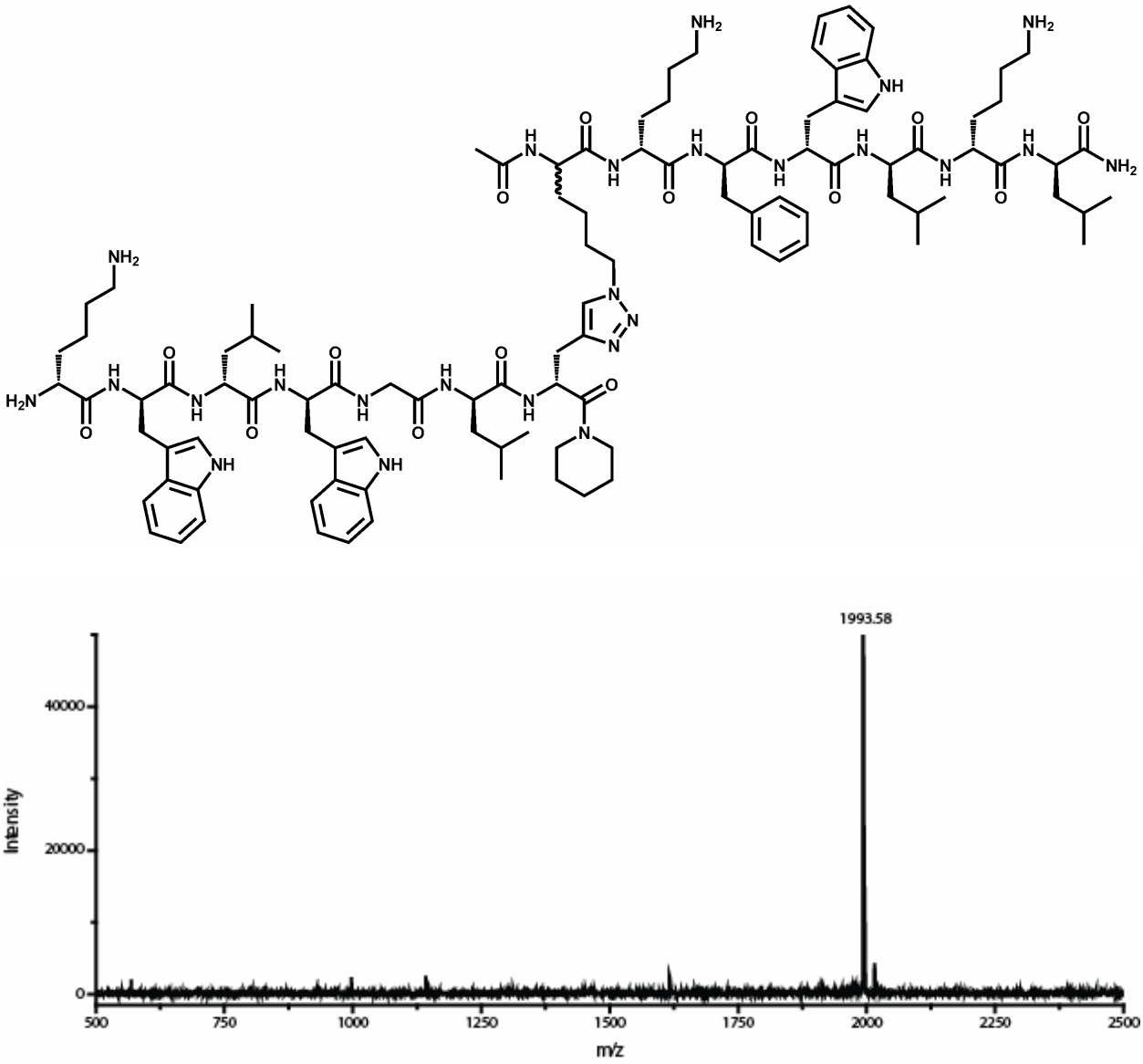


Figure S6. Biligand **kwlwGl-Tz1-kfwlkl** (Mol. Wt.: 1993.49), used in SPR measurement of affinity.

CUSTOM EDMAN DEGRADATION

To allow for resolution of artificial azide-containing amino acids by Edman degradation, the **Pulsed-Liquid cLC extended** method was utilized (Figure S7). It includes a modified gradient, **Normal 1 cLC extended** (Figure S8), and a flask cycle extended by 5 min (**Flask Normal extended**, Figure S9).

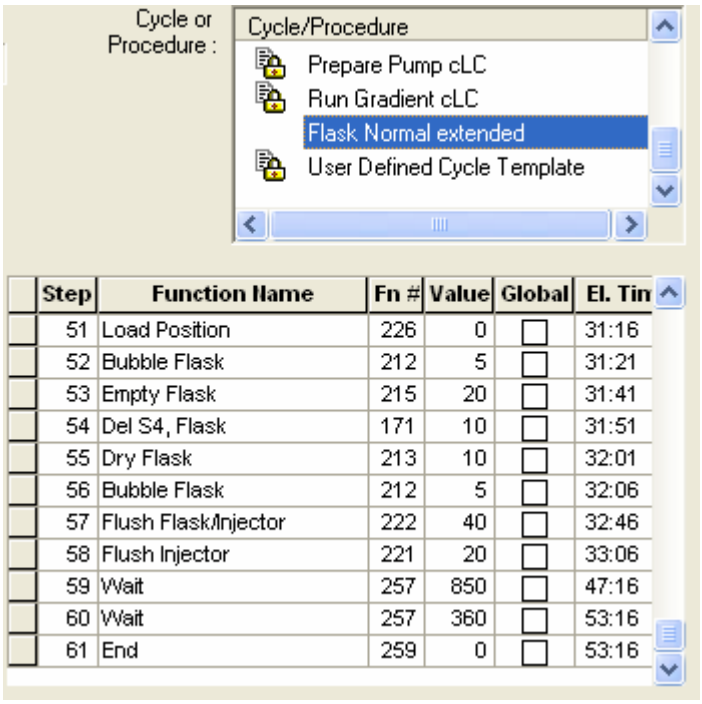
The Edman traces corresponding to elution of **Az2**, **Az4, Az6** and **Az8** are shown in Figure S10 and demonstrate a 6-min retention time increase for every two methylene units added to the azidoalkyl side chain. **Fmoc-Az2-OH** was synthesized according to literature protocol,[15] while **Fmoc-Az6-OH** was synthesized according to Scheme S1.

Cycle #	Cartridge Cycle	Flask Cycle	Gradient
Default	Cart-PL 6mmGFF cLC	Flask Normal extended	Normal 1 extended
1	None	Prepare Pump cLC	Prepare Pump cLC
2	None	Flask Blank cLC	Normal 1 extended
3	Cart Begin cLC	Flask Standard cLC	Normal 1 extended

Figure S7. Pulsed-Liquid cLC extended method.

Time	%B	uL/min	Event	Cum. Volume A	Cum. Volume B
0.0	8	40	12	0.00	0.00
0.4	12	40	1	14.40	1.60
4.0	20	40	1	135.36	24.64
22.0	45	40	1	621.36	258.64
34.0	60	40	1	849.36	510.64
35.0	90	40	1	859.36	540.64
39.0	90	60	0	883.36	756.64
40.0	50	20	0	889.36	770.64

Figure S8. Normal 1 cLC extended gradient.



Step	Function Name	Fn #	Value	Global	El. Tin
51	Load Position	226	0	☐	31:16
52	Bubble Flask	212	5	☐	31:21
53	Empty Flask	215	20	☐	31:41
54	Del S4, Flask	171	10	☐	31:51
55	Dry Flask	213	10	☐	32:01
56	Bubble Flask	212	5	☐	32:06
57	Flush Flask/Injector	222	40	☐	32:46
58	Flush Injector	221	20	☐	33:06
59	Wait	257	850	☐	47:16
60	Wait	257	360	☐	53:16
61	End	259	0	☐	53:16

Figure S9. Final steps of **Flask Normal extended** flask cycle.

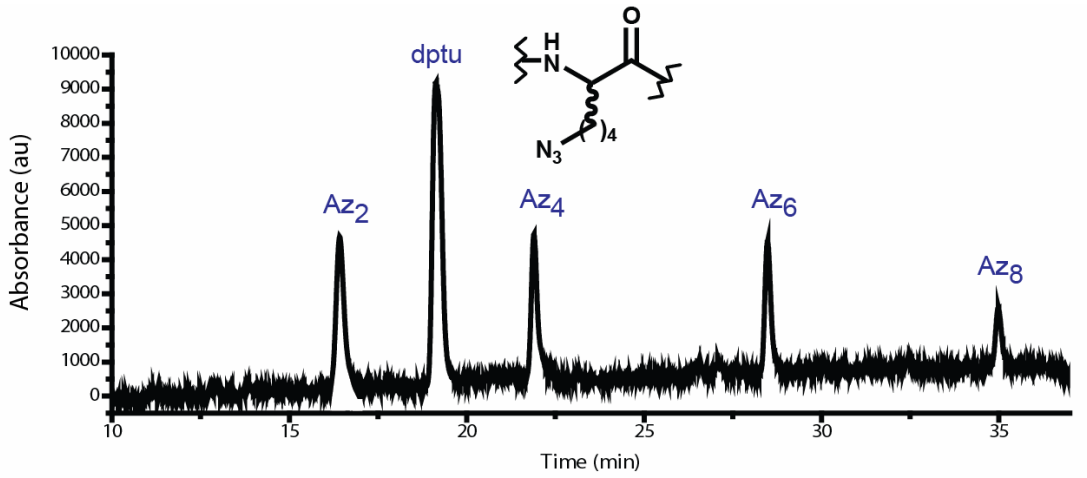


Figure S10. Edman traces for artificial azide-containing amino acids.

AFFINITY MEASUREMENTS

Fluorescence polarization. The N-terminus of the anchor ligand was labeled with fluorescein isothiocyanate (FITC) following published protocols.[16] After resin cleavage, the crude fluoresceinated anchor ligand was precipitated with ether and then purified to >98% by C_{18} reversed phase HPLC.

Luminescence spectra were recorded by Fluorolog2 spectrofluorimeter (Jobin Yvon, Longjumeau, France). All samples contained 6 μM fluoresceinated anchor ligand and varying concentrations of bCAII (0.2 μM to 800 μM) in PBS (pH 7.4) + 3% (v/v) DMSO. Stock protein and anchor ligand concentrations were verified by UV-Vis using ε_{280} (bCAII) = 57,000 $M^{-1}cm^{-1}$ or ε_{494} (FITC, 0.1 N NaOH) = 68,000 $M^{-1}cm^{-1}$ for fluoresceinated anchor ligand. Samples were excited at 488 nm (2-nm band-pass), and luminescence spectra were obtained between 500 nm and 700 nm (4-nm band-pass). All measurements were taken at 2-nm intervals with 0.5 s integration times at 25 °C. All luminescence spectra were subjected to background subtraction.

The ratio of sensitivities (G) for the vertically and horizontally plane-polarized light in the system was calculated by the equation $G=I_{HH}/I_{HV}$ using the I_{HH} and I_{HV} luminescence spectra obtained from a peptide-only sample. The luminescence spectra I_{VV} and I_{VH} were integrated, and the fluorescence polarization value (*P*) was obtained by applying Equation 1.

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \qquad (1)$$

Polarization values were fitted with a sigmoidal dose-response curve. Figure S11 shows that the anchor ligand **lklwfk-(D-Pra)** exhibited ~500 μM affinity to bCAII.

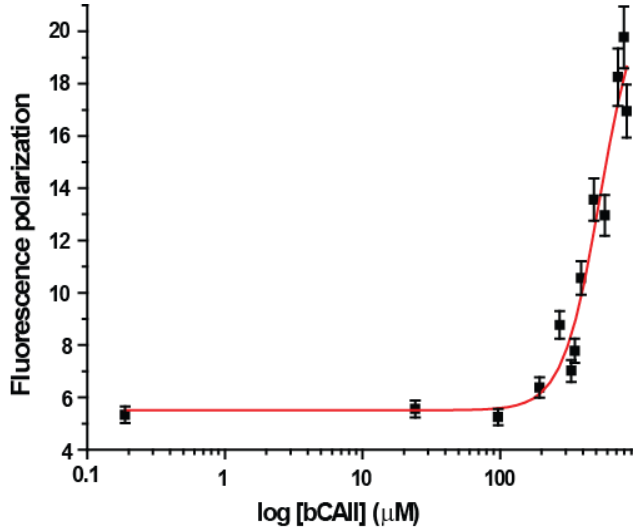


Figure S11. Fluorescence polarization binding isotherm for the anchor ligand **lklwfk-(D-Pra)**, showing $K_D \approx 500$ μM. For fluorescence polarization experiments, the anchor ligand was labeled with FITC at the N-terminus. All samples contained 6 μM FITC-anchor ligand and varying concentrations of bCAII (0.2 μM to 800 μM).

Surface plasmon resonance (SPR). These affinity measurements utilized a Biacore T100 SPR (Caltech Protein Expression Center). One flow

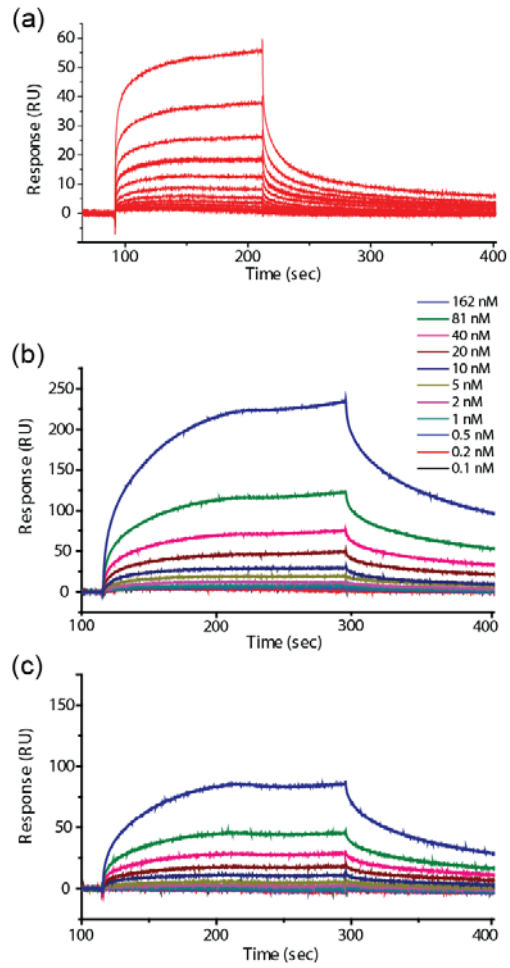


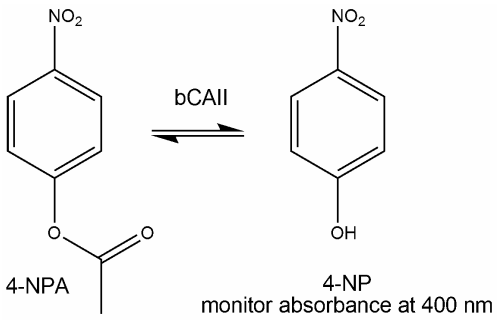
Figure S12. **(a)** SPR response sensorgrams obtained with increasing concentration of the biligand **kwlwGl-Tz1-kfwlkl** (2 nM to 5 µM) demonstrate a 3-µM biligand affinity to bCAII. For the triligand (**rfviln-Tz2-kwlwGl-Tz1-kfwlkl**), SPR response sensorgrams were obtained with increasing peptide concentration (0.1 nM to 162 nM) and demonstrate 45-nM and 64-nM affinities for human **(b)** and bovine **(c)** CA II, respectively.

cell of the biosensor surface (Biacore CM5) was immobilized with bCAII following standard procedures using 0.25 mg/mL bCAII prepared in 10 mM sodium acetate (pH 5.0) buffer and a 1:1 solution of 0.1 mM NHS and 0.4 mM EDC.[17] Similarly, a second flow cell was immobilized with hCAII following standard procedures using 0.25 mg/mL hCAII prepared in 10 mM sodium acetate (pH 5.5) buffer.[18] Immobilization levels of ~4000 RU were achieved using a flow rate of 100 µl/min over 420 s. The remaining two flow cells were left underivatized, to correct for changes in bulk refractive index and to assess non-specific binding. The running buffer was prepared to contain 10 mM HEPES + 150 mM NaCl + 0.05% Tween20 + 3% DMSO, and this buffer was used for all experiments.

Prior to the peptide analyte experiment, 8 buffer-alone cycles were completed to establish baseline stabilization. Response data were then collected for anchor, biligand, or triligand peptide samples over varying concentrations at 100 µL/min flow rate, 120-180 s contact time, and 300 s dissociation phase across the four flow cells. Following background subtraction, the analyte response data was fitted for 1:1 binding affinity using the BiaEvaluation software. In Figure S12, representative results are shown for biligand (a) and triligand (b, c).

ENZYMATIC ACTIVITY ASSAY IN THE PRESENCE OF TRILIGAND

The active site of bCAII possesses an intrinsic esterase activity which can be monitored spectrophotometrically. Specifically, bCAII catalyzes the hydrolysis of 4-nitrophenyl acetate (4-NPA) to 4-nitrophenol (4-NP), whose appearance can be monitored by absorbance at 400 nm.[19] The enzyme-catalyzed hydrolysis proceeds at a range of pH and serves as a test for active site binding by common inhibitors (Scheme S3). Here, this activity assay was used to determine whether the triligand capture agent interferes with bCAII esterase activity, which would suggest active site binding by the triligand. Solution assays were 200 µL in volume, containing 1.4 µM bCAII, 5 µM triligand, and 50 µM 4-NPA in Tris buffer composed of 9 mM Tris-HCl and 81 mM NaCl + 9% acetonitrile (v/v) + 1% DMSO (v/v). Control assays were conducted in the absence of triligand, and in the absence of protein. The hydrolysis of 4-NPA was monitored over a time course of 60 minutes, with absorbance measurements taken every 6 minutes.



Scheme S3. Esterase activity of bCAII, using 4-NPA as the hydrolytic substrate.

The experimental results are presented in Figure S13. We observed that there was an initial "burst" in 4-NP formation, followed by a slow increase in the product formation over the 60 min. Because there was no appreciable change in the bCAII esterase activity when the triligand capture agent was included in the assay, apparently this peptide binds to an epitope distinct from the bCAII active site.

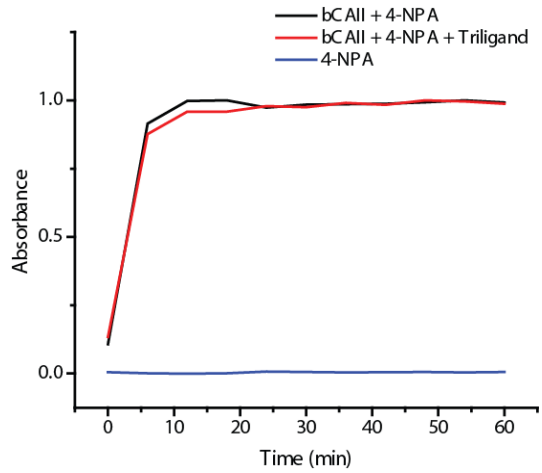


Figure S13. Enzymatic activity of bCAII in the presence of triligand. Absorbance data monitor the bCAII-catalyzed hydrolysis of 4-NPA to 4-NP (ε = 18,400 $M^{-1}cm^{-1}$ at 400 nm) at the protein active site. Experiments were performed with (red) and without (black) triligand capture agent. Additionally, an assay was performed in the presence of 4-NPA alone (blue) to determine the slow background hydrolysis of the ester in aqueous solution. [bCAII] = 1.4 µM, [Triligand] = 5 µM, and [4-NPA] = 50 µM in Tris buffer [9 mM Tris-HCl, 81 mM NaCl, 9% acetonitrile (v/v),1% DMSO (v/v)].

DOT BLOT SELECTIVITY/SENSITIVITY ASSAYS IN SERUM

The sensitivity and selectivity of the multi-ligand (biligand and triligand) capture agents for b(h)CAII in complex environments were demonstrated through the use of dot blot experiments in 10% porcine serum. For these tests, Biotin-PEG-NovaTag resin (0.48 mmol/g; Novabiochem) was utilized for bulk synthesis of C-terminal biotin-labeled multi-ligands. After resin cleavage, the crude biotinylated multi-ligand was precipitated with ether and then purified to >98% by C_{18} reversed phase HPLC.

b(h)CAII antigens were prepared as 1 mg/mL stocks in PBS (pH 7.4). A dilution series of antigen was applied to a nitrocellulose membrane, typically ranging from 2 μg to 0.5 ng per spot. The membrane was blocked at 4 °C overnight in 5% milk in Tris-buffered saline (TBS) [25 mM Tris, 150 mM NaCl, 2 mM KCl (pH 8.0)]. The membrane was then washed with TBS. The biotinylated multi-ligand was prepared at 1 μM in 10% porcine serum in TBS + 0.1% DMSO (v/v) and incubated over the membrane overnight at 4 °C. After washing with TBS for 1 h, 1:3000 Streptavidin-HRP (Abcam, Cambridge, MA) prepared in 0.5% milk/TBS was added to the membrane and incubated for 1 h. After washing with TBS for 1 h, the membrane was treated to chemiluminescent reagents (SuperSignal West Pico Chemiluminescent Enhancer and Substrate Solutions, Pierce, Rockford, IL) and then immediately developed on film.

ON-BEAD DETECTION OF IN SITU TRIAZOLE FORMATION

A biotin conjugate of the biligand anchor was prepared by modifying the N-terminus with an ethylene glycol linker (Fmoc-NH-$(PEG)_5$-COOH, EMD Biosciences) followed by biotin, by standard SPPS. A stock solution of this biotinylated biligand anchor **Biotin-$(EG)_5$-(D-Pra)-kwlwGl-Tz1-kfwlkl** (1.25 mM, alkyne) was prepared in DMSO (EG = ethylene glycol). Stock solutions of bCAII (30 μM) and hCAII (30 μM) were prepared in 50 mM Tris-Cl buffer (pH 7.2). For control experiments, stock solutions of human transferrin (Tf, 30 μM) and BSA (30 μM) were prepared in 50 mM Tris-Cl buffer (pH 7.2), and **Biotin-RPRAAA-Pra** (1.25 mM, alkyne with no documented affinity for CA II) was prepared in DMSO. The consensus 3° ligand **Az4-nlivfr** (azide) was synthesized in bulk on TentaGel S-NH_2 beads. Each in situ reaction contained 0.5 mg beads appended with 3° ligand, 30 μM biotinylated peptide-alkyne, and 15 μM protein in a final volume of 50 μL 50 mM Tris-Cl buffer (pH 7.2) + 2.5% DMSO (v/v). In situ click reactions proceeded for 24 h at 25 °C with shaking. Reactions were quenched with 50 μL

7.5 M guanidine hydrochloride (GuHCl, pH 2.0). Following incubation with GuHCl (pH 2.0) for 1 h, the beads were washed with 10 × 200 μL water, leaving only covalently bound peptides (3° ligand and biotinylated in situ triligand) on the bead.

To prepare for the enzyme-linked, colorimetric assay,[20] beads were washed with 3 × 100 μL Blocking Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 150 mM NaCl, 14 mM 2-mercaptoethanol, 0.1% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.5). Beads were then incubated in Blocking Buffer for 1 h with shaking. Alkaline phosphatase-streptavidin (AP-SA, Promega) was introduced at 1:300 dilution in Blocking Buffer to bind to any potential bead-bound biotinylated triligand. This AP-SA solution was incubated for 1 h with shaking. Excess AP-SA was then removed by washing the beads with 3 × 300 μL Wash 1 Buffer (25 mM Tris-Cl, 10 mM $MgCl_2$, 150 mM NaCl, 14 mM 2-mercaptoethanol, pH 7.5), followed by 2 × 250 μL Wash 2 Buffer (25 mM Tris-Cl, 14 mM 2-mercaptoethanol, pH 7.5). Beads were developed for 2 h in 50 μL of the chromogenic substrate BCIP (5-bromo-4-chloro-3-indoyl phosphate, Promega).

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